Hygienic Behavior of Cape and European *Apis mellifera* (Hymenoptera: Apidae) toward *Aethina tumida* (Coleoptera: Nitidulidae) Eggs Oviposited in Sealed Bee Brood

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ABSTRACT In this study, we tested for the presence and efficacy of hygienic behavior by Cape honey bees in South Africa and European honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), of mixed origin in the United States toward *Aethina tumida* Murray (Coleoptera: Nitidulidae) eggs oviposited in sealed bee brood. We looked for colony differences in removal rates of brood in cells with cappings perforated by *A. tumida* within each subspecies to identify colonies within location that display superior hygienic behavior. Finally, we determined the oviposition rate (number of *A. tumida*-perforated cells actually oviposited in by *A. tumida*/total number of *A. tumida*-perforated cells) in *A. tumida*-perforated cells and the number of *A. tumida* eggs oviposited in each cell. There were no colony differences within subspecies for the removal of normal capped brood, artificially perforated brood (capped cells perforated by experimenter with a pin), and *A. tumida*-perforated brood. For both subspecies, the bees removed significantly more *A. tumida*-perforated brood than either normal or artificially perforated brood. *A. tumida* oviposited significantly more eggs per cell in Cape colonies than in European colonies, but the oviposition rate in *A. tumida*-perforated cells did not differ between Cape and European colonies. Both subspecies removed a proportion of *A. tumida*-perforated brood statistically indistinguishable from the proportion of *A. tumida*-perforated brood containing *A. tumida* eggs. Thus, both Cape and European *A. mellifera* preferentially remove the contents of *A. tumida*-perforated cells in which *A. tumida* have actually oviposited.

KEY WORDS *Aethina tumida*, hygienic behavior, oviposition, Cape honey bees, European honey bees

HONEY BEES, *Apis mellifera* L., express hygienic behavior, which is defined as the detection of abnormal brood, removal of the wax covering it, and removal of the affected larva or pupa, a behavior generally understood to be a defensive strategy against a host of parasites and pathogens (Boecking and Spivak 1999, Spivak and Boecking 2001). Rothenbuhler (1964), who advanced the study of hygienic behavior, demonstrated that European *A. mellifera* can detect and remove brood killed by *Paenibacillus larvae* White, and others have subsequently shown detection and removal of brood affected by *Ascosphaera apis* Maassen ex Claussen and *Varroa destructor* Anderson & Trueman (Gilliam et al. 1983, Spivak and Gilliam 1993, Boecking and Spivak 1999, Spivak and Boecking 2001).

Female small hive beetles, *Aethina tumida* Murray, oviposit in bee brood cells capped with wax (Ellis et al. 2003a,b), and the removal of this brood may be one component that contributes to the overall success of natural host colonies (African subspecies of *A. mellifera*) at limiting *A. tumida*-associated depredation (Ellis et al. 2003b). Failure to remove brood in which *A. tumida* have oviposited could easily lead to a population buildup of *A. tumida* larvae (we have found as many as 120 *A. tumida* eggs oviposited in one brood cell), which in turn damage host colonies by consuming honey, pollen, and bee brood (Elzen et al. 1999, Hood 2000, Ellis et al. 2002).

In this study, we tested for the presence and efficacy of hygienic behavior by Cape honey bees, *Apis mellifera capensis* Esch., in South Africa and European *A. mellifera* of mixed origin in the United States toward *A. tumida* eggs oviposited in sealed bee brood. We set forth a practical assay that can be used to test for the presence and degree of hygienic behavior toward *A. tumida* eggs expressed by a single *A. mellifera* colony. We also looked for colony differences within each bee subspecies for the removal rates of brood cells perforated by *A. tumida* to possibly identify colonies within each location that display superior hygienic behavior. Finally, we determined the oviposi-

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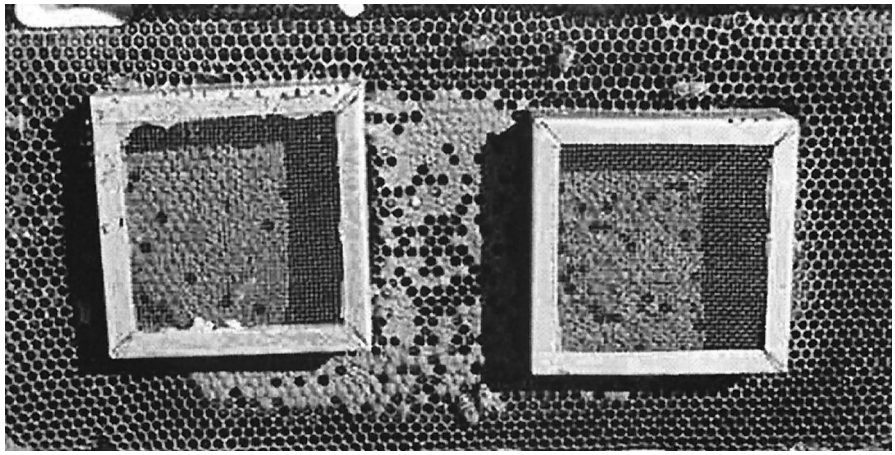

Fig. 1. Metal push-in cages used to confine adult *A. tumida* to sections of brood. The face of the cage was screen mesh (for ventilation). For each experimental replicate, one cage contained *A. tumida* and the other cage remained empty.

tion rate in *A. tumida*-perforated cells (number of *A. tumida*-perforated cells in which *A. tumida* actually oviposited/total number of *A. tumida*-perforated cells) and the number of *A. tumida* eggs oviposited in each cell.

Materials and Methods

Experiments on Cape *A. mellifera* were conducted at a Rhodes University research apiary outside of Grahamstown, South Africa (a geographic area predominantly inhabited by Cape bees) in March through May 2003. The complimentary studies with European *A. mellifera* of mixed origin were conducted at The University of Georgia research apiary, Oconee County, in July and August 2003. Ten colonies of Cape *A. mellifera* and nine colonies of European *A. mellifera* (housed in standard Langstroth-style hives of equal strength and having nearly identical reserves of brood, honey, pollen, and adult bees) were used for the study. All colonies had been previously and naturally exposed to *A. tumida.*

We established three experimental treatments: capped brood that had been 1) perforated by *A. tumida*, 2) artificially perforated by experimenter (positive control), or 3) not perforated (negative control). This was accomplished by trapping *A. tumida*, or excluding them, on a 10 by 10-cm area of sealed brood with a sheet metal push-in cage (10 by 10 by 2.5 cm), the face of which was screened to allow for ventilation but exclude bees and other *A. tumida* (Fig. 1). The combs contained ≈60–90% capped brood. The selected brood was >6 d from eclosing (determined by uncapping and examining brood in the test area) so that no brood from the test area would emerge during the study. For each colony, the frame of capped brood was removed, and 20 adult *A. tumida* (nonsexed, captured from nature or laboratory-reared, cooled in a vial surrounded by ice for 4–5 min) were placed under one cage (the adults mate and the females subsequently oviposit); this prepared the *A. tumida*-perforated treatment. A second cage without *A. tumida* was pushed into the same brood frame as a nonperforated negative control. Both caged sections of brood were then returned to the center of the bee cluster in each colony.

Twenty-four hours later, both cages were removed, and adult *A. tumida* from the treatment cage were collected. Cells containing *A. tumida* perforations (Ellis et al. 2003a) in the *A. tumida*-perforated treatment square were counted and labeled by placing a transparent sheet of acetate over the brood and marking all cells having perforated cappings. Similarly, 20 nonperforated brood cells (no perforations in the cappings) from under the negative control cage were marked. The positive control (artificial perforations) was created by puncturing the cappings of 20 brood cells with a minuten insect pin to simulate *A. tumida* oviposition perforations. The perforations were positioned around the capping perimeter to avoid damaging the pupae (pin-killed pupae are removed by bees; Boecking and Spivak 1999). The documented brood cells of all three treatments were then returned to the center of the bee cluster. After 48 h, they were removed and marked cells from which brood had been removed by the bees were counted. The procedure was replicated three times for each Cape and European colony.

The oviposition rate in *A. tumida*-perforated cells also was determined. For each of six Cape and seven European colonies, 20 adult *A. tumida* were confined to one frame of capped brood as described above, and the frames were returned to the colonies. Twenty-four hours later, cells with perforations in their cappings were opened to determine the presence or absence of *A. tumida* eggs (≈30 cells per colony in Cape colonies were opened, and all perforated cells in European colonies were opened). The oviposition rate was calculated as the percentage of *A. tumida*-perforated cells actually containing *A. tumida* eggs. The number of *A. tumida* eggs was determined for each cell in which oviposition occurred.

Table 1. Colony removal rate (proportion) of *A. mellifera* brood cells that were non-perforated (negative control), artificially-perforated (positive control), or *A. tumida*-perforated

Colony	Cape *A. mellifera*			European *A. mellifera*		
	Non-perforated	Artificially-perforated	*A. tumida*-perforated	Non-perforated	Artificially-perforated	*A. tumida*-perforated
1	0.02 ± 0.02	0.02 ± 0.02	0.41 ± 0.14	0.03 ± 0.03	0.15 ± 0.09	0.59 ± 0.10
2	0.03 ± 0.02	0	0.73 ± 0.13	0.02 ± 0.02	0.08 ± 0.08	0.73 ± 0.03
3	0	0	0.74 ± 0.14	0	0.12 ± 0.04	0.67 ± 0.03
4	0	0.02 ± 0.02	0.71 ± 0.07	0.02 ± 0.02	0.25 ± 0.18	0.51 ± 0.08
5	0.03 ± 0.02	0.02 ± 0.02	0.57 ± 0.15	0.02 ± 0.02	0.23 ± 0.21	0.51 ± 0.12
6	0.08 ± 0.04	0.02 ± 0.02	0.79 ± 0.14	0	0.07 ± 0.07	0.42 ± 0.12
7	0	0.05 ± 0.03	0.67 ± 0.11	0	0.10 ± 0.08	0.58 ± 0.08
8	0.10 ± 0.08	0.02 ± 0.02	0.69 ± 0.07	0	0.03 ± 0.02	0.60 ± 0.10
9	0.07 ± 0.07	0.02 ± 0.02	0.65 ± 0.05	0	0.08 ± 0.04	0.46 ± 0.09
10	0.07 ± 0.04	0.03 ± 0.02	0.71 ± 0.12			

Colonies within each subspecies did not differ with respect to the amount of brood removed within each treatment type. Data are mean ± standard error, $n = 3$ for all data. Data within columns are not different at the $\alpha \leq 0.05$ level.

Statistical Analyses. Differences between colony removal rates of *A. tumida*-perforated, nonperforated (negative control), and artificially perforated (positive control) brood were analyzed within bee subspecies by using one-way analysis of variance (ANOVA). Because colonies within subspecies did not differ with respect to the amount of treatment brood removed (i.e., no colonies within subspecies were "more hygienic" than others), colony replicates were averaged (=proportion of brood removed) for each colony for use in further analyses. The proportion of brood removed was analyzed by ANOVA recognizing treatment and *A. mellifera* subspecies (Cape or European) as main effects. Because there was an interaction between treatment and subspecies, the proportion of brood removed was analyzed further by subspecies by using ANOVA. Differences in the oviposition rate in perforated cells and in the number of *A. tumida* eggs per cell were analyzed by *A. mellifera* subspecies by using independent sample *t*-tests. Furthermore, the oviposition rate in perforated cells was compared with the removal rate of perforated cells for both subspecies by using independent sample *t*-tests. Where analyzed data were proportions (as in the proportion of removed brood and the oviposition rate), data were transformed using arcsine $\sqrt{}$proportion to stabilize the variance before analyses. All differences were accepted at $\alpha \leq 0.05$, and all analyses were conducted using Statistica (2001).

Results

Colony-Level Removal of Perforated Brood. There were no colony differences among Cape *A. mellifera* for the removal of nonperforated ($F = 1.1$; df = 9, 20; $P = 0.4364$), artificially perforated ($F = 0.6$; df = 9, 20; $P = 0.7510$), or *A. tumida*-perforated ($F = 0.8$; df = 9, 20; $P = 0.6602$) brood. Furthermore, there were no colony differences among European *A. mellifera* for the removal of nonperforated ($F = 0.6$; df = 8, 18; $P = 0.7359$), artificially perforated ($F = 0.3$; df = 8, 18; $P = 0.9373$), or *A. tumida*-perforated ($F = 1.2$; df = 8, 18; $P = 0.3647$) brood. Mean removal rates for colonies of both bee subspecies are reported in Table 1.

Hygienic Behavior of Cape and European Bees. There were no subspecies effects for the total proportion of brood removed ($F = 0.1$; df = 1, 51; $P = 0.7716$). Overall, Cape bees removed the same proportion of all tested brood (0.24 ± 0.06, 30; mean ± SE, *n*) as did their European counterparts (0.23 ± 0.05, 27). There were treatment effects ($F = 336.4$; df = 2, 51; $P < 0.0001$) and treatment × subspecies interactions ($F = 16.9$; df = 2, 51; $P < 0.0001$) for the proportion of brood removed. Because of the significant interaction, the removal data were analyzed separately by subspecies. There was a significant difference in the amount of treatment brood removed within both Cape ($F = 202.8$; df = 2, 27; $P < 0.01$) and European ($F = 152.4$; df = 2, 24; $P < 0.0001$) *A. mellifera.* For both subspecies, the bees removed significantly more *A. tumida*-perforated than either nonperforated or artificially perforated brood (Table 2). In Cape colonies, the amount of nonperforated and artificially perforated brood did not differ, whereas it did in European colonies (Table 2). Colonies of both bee subspecies also uncapped some *A. tumida*-perforated pupae (<5%) without removing them.

Oviposition Rate and Number of Eggs per Cell. There was no difference between Cape and European *A. mellifera* for the oviposition rate in cells perforated by *A. tumida* ($t = 1.5$, df = 11, $P = 0.1642$). In Cape colonies, the proportion of *A. tumida*-perforated cells in which *A. tumida* oviposited (0.68 ± 0.04; 6) was

Table 2. Removal rate (proportion) of *A. mellifera* brood cells that were non- perforated (negative control), artificially-perforated (positive control), or *A. tumida*-perforated

Treatment	Cape *A. mellifera*	European *A. mellifera*
Non-perforated	0.04 ± 0.01a	0.01 ± 0.004a
Artificially-perforated	0.02 ± 0.005a	0.12 ± 0.02b
A. tumida-perforated	0.67 ± 0.03b	0.57 ± 0.03c

Data were analyzed by subspecies, because of the significant interaction between treatment and and *A. mellifera* subspecies. Data are mean ± standard error. Ten Cape and nine European colonies were sampled. Columnar data followed by the same letter are not different at the $\alpha \leq 0.05$ level.

similar to that in European colonies (0.56 ± 0.06; 7). *A. tumida* oviposited significantly more eggs per cell in Cape colonies (14.5 ± 1.4; 122) than in European colonies (7.3 ± 0.4; 312) ($t = 7.0$, df = 432, $P < 0.0001$). In Cape colonies, the proportion of *A. tumida*-perforated brood in which *A. tumida* oviposited was not significantly different from the proportion of *A. tumida*-perforated brood that was removed by the bees ($t = 0.2$, df = 14, $P = 0.8367$); the same held true in European colonies ($t = 0.1$, df = 14, $P = 0.9393$).

While rearing *A. tumida* in vitro for use in this study, we observed the process by which *A. tumida* perforate and oviposit in capped brood cells. Female *A. tumida* use their mandibles to bite small holes through the cell capping. They then position the distal terminus of their abdomen flush with the perforation and insert their ovipositor to begin laying eggs. This process usually lasts >5 s per occurrence, probably depending on the number of eggs the females were ovipositing per cell.

Discussion

In colonies of European species of *A. mellifera*, *A. tumida* perforate cell cappings and oviposit even in the presence of bees (Ellis et al. 2003a), but it is not yet known whether they do the same in colonies of African subspecies of *A. mellifera.* This mode of oviposition may be an important reproductive pathway for *A. tumida* (Ellis et al. 2003b), because exposed *A. tumida* eggs are removed quickly from colonies (Neumann and Härtel 2004). Lundie (1940) and Schmolke (1974) suggest that *A. tumida* oviposit in cracks and crevices around the hive. However, this would require hatching larvae to crawl to the combs while evading bees, and studies have shown that free-roaming larvae are removed from African colonies (Neumann and Härtel 2003). Therefore, direct oviposition into brood cells may be a superior survival strategy (Ellis et al. 2003b). As a result, the hygienic removal of brood on which *A. tumida* oviposits may be an important resistance mechanism against this nest invader.

The data indicate that both Cape and European *A. mellifera* remove brood on which *A. tumida* have oviposited. If this behavior were essential to the resistance of Cape bees toward *A. tumida* depredation, then one would expect to find the behavior reduced or absent in European bees. This was not the case. It remains possible that subspecific differences with respect to the removal rate of *A. tumida*-perforated brood will emerge if larger areas of brood are involved.

Interestingly, both subspecies removed the same proportion of *A. tumida*-perforated brood as that in which *A. tumida* actually oviposited, a finding similarly demonstrated for a second mode of *A. tumida* oviposition wherein *A. tumida* enter empty cells and oviposit through the cell wall into an adjacent cell (Ellis et al. 2003b). In the current study, both subspecies removed an amount of *A. tumida*-perforated brood equal to that of the actual oviposition rate, suggesting that they preferentially open and remove brood from those perforated cells actually containing eggs. Furthermore, neither subspecies removed artificially perforated brood at similar or higher rates than *A. tumida*-perforated brood, suggesting that it is not the perforated capping that stimulates the removal of cell contents.

The stimuli that elicit removal of *A. tumida* egg-infested cells remain unclear. Pathogen-killed brood may be recognized and removed by bees (Rothenbuhler 1964, Boecking and Spivak 1999); however, the oviposition tactics of *A. tumida* may not necessarily kill the brood. Despite this, both bee subspecies were able to detect and remove brood on which *A. tumida* had oviposited. One possibility is that the presence of *A. tumida* eggs or an unknown oviposition chemical deposited by female *A. tumida* causes bees to remove the cell contents. Also possible is that because *A. tumida* eggs can hatch within 48 h (Schmolke 1974), the beetle larvae damage the bee pupae or secrete a substance that elicits the bees to remove the cell contents.

If bees cue onto the presence of *A. tumida* eggs, there may exist a minimum number of eggs per cell that elicits the removal of the cell contents. If so, then one would expect that colonies in which *A. tumida* lay fewer eggs per cell would be less likely to detect and remove infested brood. This study does not permit one to determine whether such a putative egg threshold exists, but *A. tumida* clearly laid fewer eggs per cell in European colonies, perhaps increasing the bees' chances of missing infested cells in these colonies. As a result, putting fewer *A. tumida* under each cage may encourage *A. tumida* to oviposit fewer eggs per cell, because competition for oviposition sites could lead to the high number of eggs per cell seen in this study. Using fewer adults may make the test more sensitive to detecting differences in the removal rates between both subspecies if such differences exist.

It is also unclear why *A. tumida* perforate some cells but do not oviposit in them. In Cape colonies, ≈32% of *A. tumida*-perforated cells did not contain *A. tumida* eggs, the corresponding number for European colonies was ≈44%. This may indicate that *A. tumida* cue onto certain developmental stages of the brood or chemicals produced by the brood. Interestingly, the oviposition rate of *A. tumida*-perforated cells in Cape colonies was higher than that in European colonies. This may indicate the absence/reduction of a chemical oviposition-stimulant in non-native hosts.

One objective of this study was to determine whether colonies differed with respect to the degree of hygienic behavior they express; colony variation for hygienic removal of varroa is often high (Boecking and Spivak 1999). However, differences in the level of hygienic removal of *A. tumida*-perforated brood for colonies of either subspecies were not detected. Because other factors (such as genetics, environmental conditions, and colony size) affect hygienic expression (Boecking and Spivak 1999), one may need to control for these when trying to determine whether the level of hygienic expression toward *A. tumida* oviposition varies between colonies.

Regardless, it is interesting that all tested colonies of both bee subspecies removed *A. tumida*-perforated brood, especially because reports indicate that only few colonies (<10%) in nature express hygienic behavior (Boecking and Spivak 1999). This further suggests that the level of removal stimulants in the brood (such as eggs and oviposition chemicals) in our study may have been unnaturally high. This demonstrates a need to examine *A. tumida* stimuli that elicit brood removal so that one may manipulate these factors experimentally. If successful, it may be possible to 1) further determine whether the expression of removal of *A. tumida*-perforated brood differs between African and European subspecies of *A. mellifera* and 2) select for this behavior as a natural defense against *A. tumida* depredation.

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